

ING4 induces G2/M cell cycle arrest and enhances the chemosensitivity to DNA-damage agents in HepG2 cells [☆]

Xin Zhang^{a,b,1}, Lu-Sheng Xu^{b,c,1}, Zhi-Qin Wang^b, Ke-Sheng Wang^b, Na Li^b, Zhi-Hong Cheng^b, Shang-Zhi Huang^c, Dong-Zhi Wei^a, Ze-Guang Han^{b,*}

^aState Key Laboratory of Bioreactor Engineering, New World Institute of Biotechnology, East China University of Science and Technology, Shanghai 200237, China

^bChinese National Human Genome Center at Shanghai, 351 Guo Shou-Jing Road, Shanghai 201203, China

^cDepartment of Medical Genetics, Institute of Basic Medical Sciences, CAMS, School of Basic Medicine, PUMC, Beijing, China

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Abstract The known members of inhibitor of growth (ING) gene family are considered as candidate tumor suppressor genes. ING4, a novel member of ING family, is recently reported to interact with tumor suppressor p53, p300 (a major component of histone acetyl transferase complexes), and p65(RelA) subunit of NF- κ B. In this study, we investigated the cellular behaviors of HepG2 cells with exogenous ING4. Interestingly, the overexpression of ING4 negatively regulated the cell growth with significant G2/M arrest of cell cycle, and moreover, enhanced the cell apoptosis triggered by serum starvation in HepG2 cells. Furthermore, the exogenous ING4 could upregulate endogenous p21 and Bax in HepG2 cells, not in p53-deficient Saos-2 cells, suggesting that G2/M arrest induced by ING4 could be mediated by the increased p21 expression in a p53-dependent manner, although there is no significant increase of p53 expression in HepG2 cells. Moreover, HepG2 cells with exogenous ING4 could significantly increase cell death, as exposed to some DNA-damage agents, such as etoposide and doxorubicin, implying that ING4 could enhance chemosensitivity to certain DNA-damage agents in HepG2 cells.

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1. Introduction

ING1, as the first member of the inhibitor of growth (ING) gene family, was identified by subtractive hybridization and the subsequent selection of transforming ‘genetic suppressor elements’ [1]. The described ING1 was renamed as ING1b, since two additionally alternative splicing variants of ING1 gene were found [2,3]. Interestingly, the previous studies indicated that the expression of ING1 could be significantly involved in the regulation of apoptosis and cell cycle in a p53-dependent manner in several cancers and cell lines, suggesting

that ING1 could be a novel candidate tumor suppressor [1,4–11].

Other members of the ING gene family, ING2/ING1L, ING3, ING4 and ING5, were also isolated and characterized [12–17]. Like ING1b, ING2 could negatively regulate cell proliferation in a p53-dependent manner through induction of G1 phase arrest of cell cycle and apoptosis [12]. ING3 could activate p53-transactivated promoters, including promoters of p21 and Bax, and induce a decreased population of cells in S phase and apoptosis [13]. ING4, described as ING1 homolog (GenBank Accession Nos. AF110645 or NM_016162), was isolated from human pituitary by our previous works [16]. In addition, Shiseki et al. [17] also described some characteristics of ING4 and ING5, which the transient overexpression of ING4 or ING5 could induce a decreased cell population in S phase of cell cycle and apoptosis in a p53-dependent manner, along with the increased p21 expression in RKO cells. Moreover, ING4 could physically interact with p300 and p53 in vivo, as well as enhance p53 acetylation at Lys-382. Most interestingly, Garkavtsev et al. [18] reported that ING4 was involved in regulating brain tumor growth and angiogenesis by associating with p65(RelA) subunit of NF- κ B. In this study, we first found that ING4 could regulate negatively the cell growth with significant G2/M arrest of cell cycle and enhance chemosensitivity to certain DNA-damage agents in HepG2 cells.

2. Materials and methods

2.1. Plasmids constructs

The entire open reading frame of human ING4 was subcloned into mammalian cell expression vectors pcDNA3.0 (Invitrogen) and pcDNA3.1-cMyc-his (Invitrogen). pGEX5x-1-ING4 was constructed to produce the GST-ING4 fusion protein for generating antibody against human ING4.

2.2. Cell culture

HepG2 cells were cultured in MEM with 10% fetal bovine serum. 2×10^5 HepG2 cells were plated in 35-mm dishes and cultured at 37 °C for 24 h, and then were transfected with 5 μ g of pcDNA 3.0-ING4, pcDNA-3.1-ING4-cMyc-his or control plasmids, respectively, by using ProFectin Mammalian Transfection System (Promega). After 48 h, we split the transfected cells into 100-mm dishes by 1:10 dilution and subsequently screened by G418 for three weeks. The single cell clones were picked, and then verified by reverse transcriptase-PCR and western blot analysis. Saos-2 cells were cultured in McCoy’s 5a medium

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* Corresponding author. Fax: +86-21-50800402.
E-mail address: hanzg@chgc.sh.cn (Z.-G. Han).

¹ These two authors contributed equally to this work.

with 15% fetal bovine serum, and transiently transfected with pcDNA 3.0 and pcDNA3.0-ING4, respectively, by LipofectAMINE (Invitrogen). After 48 h, the cell lysates were collected for western blot analysis.

2.3. Cell proliferation, cell cycle and flow cytometry analysis

To observe the cell proliferation, the stable HepG2 subclones with overexpression of ING4 were seeded in 96 well plate at 5×10^3 cells per cm^2 and cultured for 7 days. Cell viability was measured by MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] dye reduction assay (Promega) during the time course according to the manufacturer's instructions. Simultaneously, the direct cell counting was also performed at the same time points in 24-well plates. To analyze the cell cycle, the derived HepG2 cells were harvested prior to confluence, and fixed with 70% (vol/vol) ethanol, and then re-suspended in PBS buffer containing 20 $\mu\text{g}/\text{ml}$ propidium iodide and 500 $\mu\text{g}/\text{ml}$ RNase A. The DNA contents were measured as the propidium iodide signal to determine cell cycle profiles using FACSscan (Becton–Dickinson) and then analyzed by motFIT software. At least 10 000 cells were analyzed from each sample. To evaluate the chemosensitivity of HepG2 cells to some DNA-damage agents, the cells were plated into 24-well plate at 1×10^4 cells per cm^2 , and subsequently treated with doxorubicin (DOX) and etoposide (ETO), respectively. The cell cycle profiles of the treated cells were analyzed after 48 h and the Sub-G1 population was considered as death cells. All experiments were repeated at least thrice.

2.4. Tumorigenicity of HepG2 cells constitutively overexpressing ING4

Female athymic BLAB/cA nu mice, 5–6 weeks of age, were obtained from SIPPR-BK Experimental Animal Co. (Shanghai, China) and housed in a pathogen-free facility. 5×10^6 cells with overexpression of ING4 were injected subcutaneously into the right flank of nude mice. Tumor growth was measured using caliper every 3 days and the tumor volumes were calculated according to the formula: $\text{volume} = \text{length} \times (\text{width}^2)/2$. The tumorigenic experiments in vivo were repeated twice with four mice each group.

2.5. Detection of apoptotic cells

HepG2 cells with stable overexpression of ING4 were plated on coverslips and cultured overnight to allow the cells attaching to the coverslips. The cells were further cultured for 4 and 5 days in serum free medium and the apoptotic cells were detected with Annexin V-FITC binding assay (BD Bioscience) [19]. Simultaneously, the DAPI staining on the cells was also employed to quantitate the percent of apoptotic cells.

2.6. Antibodies

Mouse monoclonal anti-p53 (DO-1), p21 (F-5), p27KIP1 (F-8) and actin (C-2), cyclin B1(GSN1) and CDC2 were purchased from Santa Cruz biotechnology (Santa Cruz). Rabbit polyclonal anti-ING4 antibody was raised against GST-ING4 fusion protein and purified from antiserum with agarose-linked Protein G (Amersham). The anti-GST antibody was removed by GST coupled with agarose beads.

3. Results and discussion

3.1. ING4 regulate negatively the cell growth of HepG2 cells in vitro and in vivo

The previous studies from several groups implicated that some members of the ING family could negatively regulate the cell growth through different model systems [9,12,13,17,20]. To investigate the effects of ING4 on cell proliferation, we established some stable HepG2 cell lines with constitutive overexpression of ING4. In this work, three subclones (A3, P28 and B3) with exogenous ING4, and additional one (D5) expressing Myc-tagged ING4, were screened out; meanwhile, V1 and V2 subclones with the empty vector pcDNA3.0 alone, as well as V3.1 with the empty vector pcDNA3.1-cMyc-his, were served as the negative control, respectively (Fig. 1A). These derived HepG2 subclones were seeded in 96-well plates,

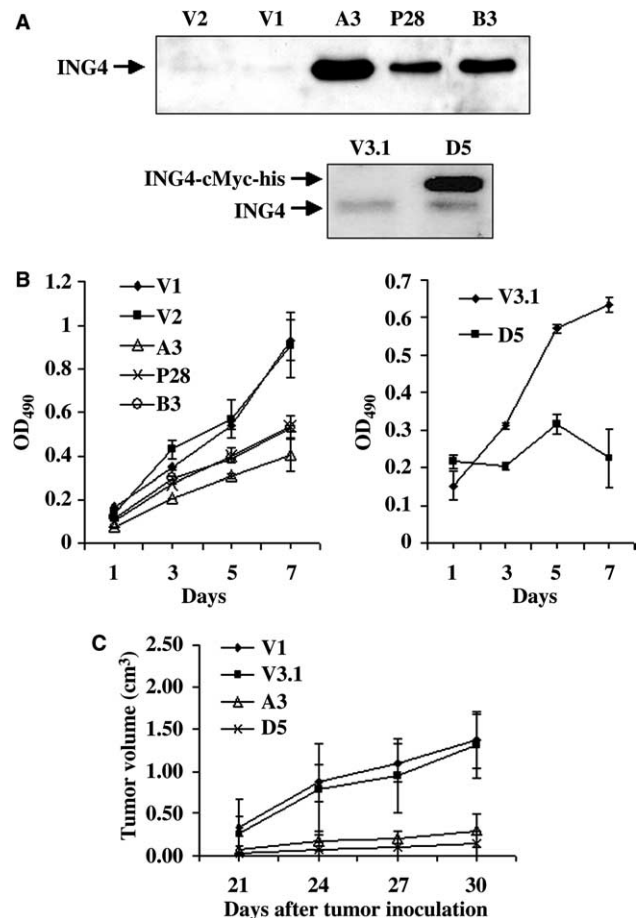


Fig. 1. The constitutive overexpression of ING4 negatively regulated the cell proliferation in HepG2 cells in vitro and in vivo. (A) Western blot analysis demonstrated the overexpression of ING4 or cMyc-his tagged ING4 in the stably transfected HepG2 cell clones, A3, P28, B3 and D5. (B) MTS assay (Promega) was employed to measure the cell viability, which indicated that HepG2 cell clones with exogenous ING4 (A3, P28, B3) or cMyc-tagged ING4 (D5) exhibited the decreased cell proliferation compared with control clones with empty vectors alone (V1, V2 and V3.1). Experiments were repeated at least thrice, and the means and S.D. are included. (C) Decreased tumorigenicity of HepG2 cells with constitutive expression of ING4 in nude mice. The tumorigenicity of A3 and D5 cells was significantly inhibited compared with that of control clones. Experiments were performed two times, four mice were used per group. ($P < 0.05$) The means and S.D. are shown for each time point.

and then the cell viability was measured by MTS assay on the cultured days, 1, 3, 5 and 7 days, respectively. Interestingly, the cell proliferation of all HepG2 clones, A3, P28 and B3, with constitutive overexpression of ING4 were significantly reduced, the cell viability was decreased as much as 45–64% on the 7th day compared with that of control clones (Fig. 1B left); and, moreover, the cell growth of D5 clone with the cMyc-tagged ING4 was more obviously inhibited than that of control clone (Fig. 1B right). The similar results were obtained by direct cell counting in the same time course (data not shown). These data suggested that the constitutive overexpression of ING4 could negatively regulate the cell growth of HepG2 cells in vitro.

To further strengthen the evidences, the tumorigenicity of stable clones A3 and D5 with exogenous ING4 was observed in nude mice by s.c. injection, as V3.1 and V1 subclones were

used as control. As expected, the tumorigenicity of A3 and D5 clones was significantly decreased as compared with control groups (Fig. 1C), suggesting that the overexpression of ING4 indeed could suppress the cell growth of HepG2 cells *in vivo*.

3.2. The overexpression of ING4 induced the G2/M arrest of cell cycle

To explore the intrinsic mechanism by which the overexpression of ING4 inhibits cell growth, cell cycle analysis was performed on the stable HepG2 clones with or without exogenous ING4. Interestingly, the significantly increased percentages of cells in G2/M phase, along with the dramatically decreased cell population of G1 phase, were observed in P28, B3, A3 and D5 clones overexpressing ING4 or cMyc-tagged ING4, not in clones with empty vector alone (Fig. 2A and B). Moreover, the extent of G2/M arrest seems to be correlated with the dose of ING4 protein in the HepG2 clones, in which A3 and D5 clones with the highest expression of ING4 exhibited the most significant G2/M arrest (Fig. 2C). These data suggested that ING4 could inhibit the cell proliferation by inducing the G2/M arrest in a dose-dependent manner in HepG2 cells through regulating the G2 checkpoint. This observation is different from the result obtained from transient overexpression of ING4 in RKO cells, which showed a decreased cell population of S phase, along with the slightly increased G1/G0 and G2/M phase [17]. The reason for why the cellular behaviors induced by exogenous ING4 were different might be due to the distinct cell lines in both studies, or the chronic effect of ING4 on HepG2 cells.

3.3. ING4 enhanced apoptosis on HepG2 cells

To evaluate the effect of ING4 on apoptosis, some stable HepG2 clones were cultured in serum-free medium for 4 and 5 days to trigger apoptosis by serum starvation. FITC-linked annexin V/propidium iodide staining assay revealed that the fraction of apoptotic cells in A3 and D5 clones was significantly greater than that in V1 and V3.1 as control (Fig. 3A and supplementary Fig. S1). Similar results were obtained by DAPI staining on the cells through counting the apoptotic cells with small and condensed nucleus (data not shown). Moreover, quantitative examination in time course indicated that the cell death was increased up to 20% in A3 and D5 clones with the stable overexpression of ING4, as compared with that of V1 and V3.1 clones as control at 4 days after serum starvation (Fig. 3B). The data suggested that ING4, as a conditional inducer, could enhance apoptosis of HepG2 cells triggered by serum starvation.

3.4. The G2/M arrest in HepG2 cells could be induced through upregulation of p21 in a p53-dependent manner triggered by ING4

The previous studies revealed that the functions of ING gene family could be correlated with p53 [9,12,13,17]. ING4 has been found to associate with p53 protein through the physical interaction and enhancing p53 acetylation at Lys-382 residues in RKO cells [17]. In this study, ING4 was proved to interact physically with p53 in HepG2 cells using the co-immunoprecipitation assay (supplementary Fig. S2). p53 can regulate the transcriptional expression of several downstream genes, such

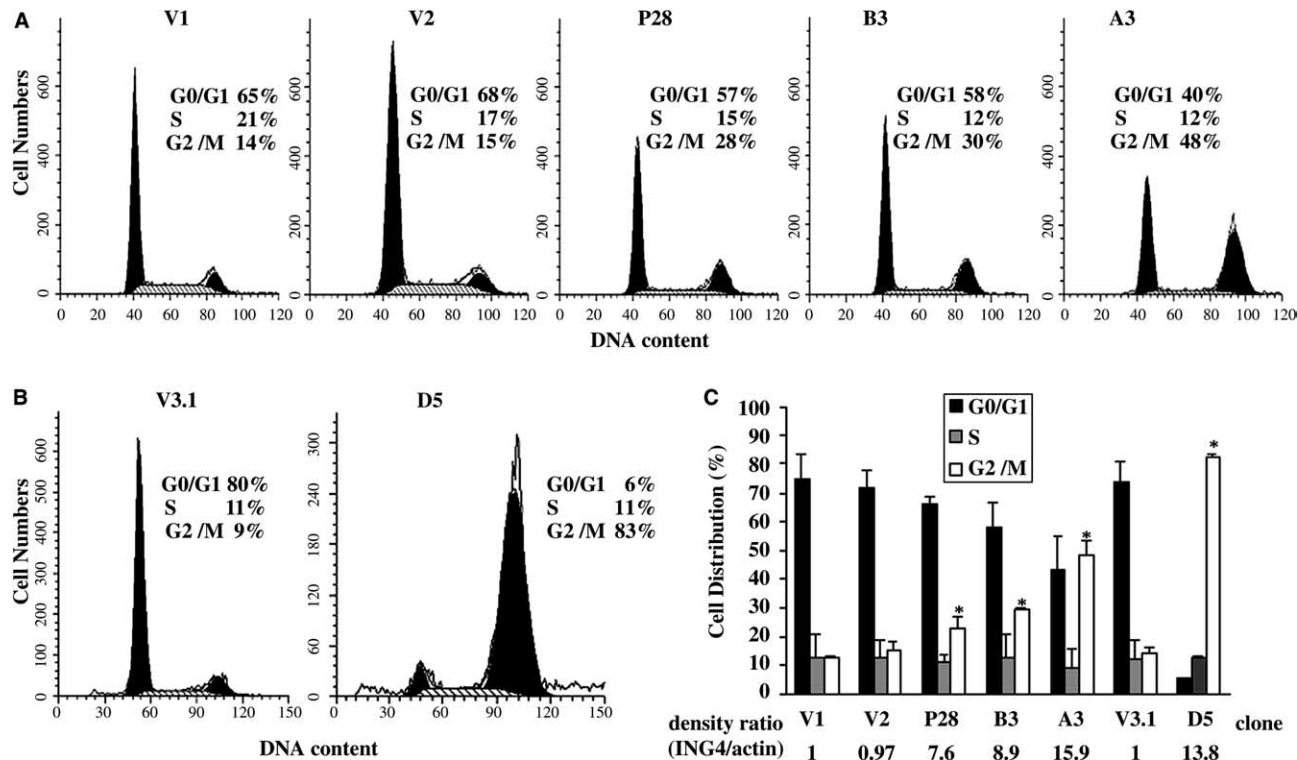


Fig. 2. Effects of ING4 on cell cycle profiles of HepG2 cells. (A) The cell cycle profiles in the HepG2 cell clones P28, B3 and A3 with ING4 overexpression. The percentages of cell population of different cell cycle phases were showed in upper-right. (B) The D5 cell clone with c-Myc-his tagged ING4 dramatically exhibited the G2/M cell cycle arrest with 83% cell population. (C) Distribution of cell population of different cell cycle phases in the HepG2 cell clone. Experiments were repeated at least three times. * $P < 0.01$ compared with control clones. Data values and error bars showed the means and S.D., respectively.

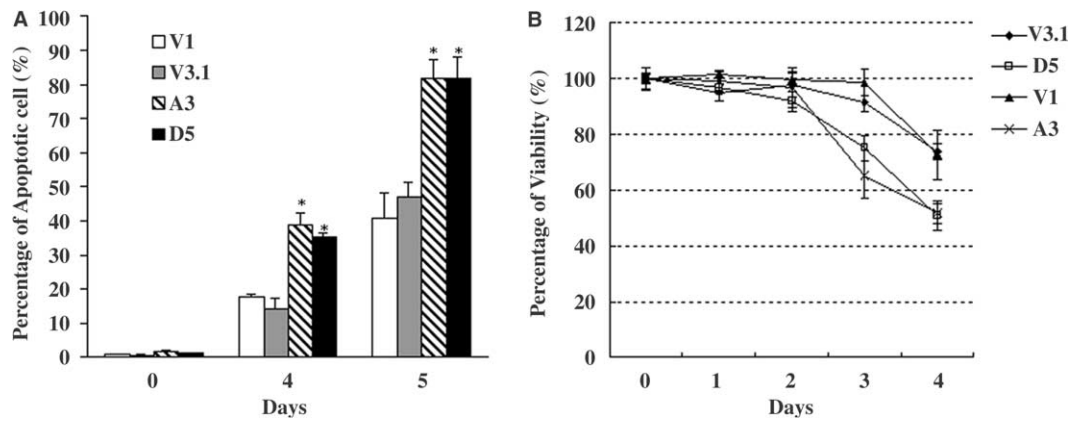


Fig. 3. Effect of ING4 on apoptosis induced by serum starvation. (A) The derived HepG2 clones, V1, A3, V3.1 and D5, were cultured in serum free medium for 4 and 5 days and stained with Annexin V-FITC/PI solution. The apoptotic cells were visualized under fluorescence microscopy. Results were indicated as the percentages of the number of apoptotic cells compared with total number of cells in five random samples. * $P < 0.01$ compared with control clones. Data are means of three independent experiments. (B) HepG2 cells of V1, A3, V3.1 and D5 were evaluated in cell culture by serum free medium for 0, 1, 2, 3 and 4 days as time course. Cell viability was measured using MTS assay. Experiments were repeated at least three times, and the means and S.D. are included.

as p21, GADD45 and Bax, which involved in cell cycle and apoptosis. Northern blotting and western blotting were employed to evaluate the transcriptional and translational levels of these genes on the stably transfected HepG2 cells. The Northern blot analysis on A3 cells showed that the transcripts of p21 and Bax were significantly increased although the transcriptional level of p53 was not obviously regulated by

ING4 (Fig. 4A). However, GADD45, which has been reported to play a critical role in G2/M arrest induced by ultraviolet light or methyl methanesulfonate [21], was not increased. Furthermore, Western blot analysis revealed that p21 protein was significantly elevated in the HepG2 subclones with exogenous ING4, not along with the increase of p53 protein (Fig. 4B). Interestingly, the upregulation of p21 seems to

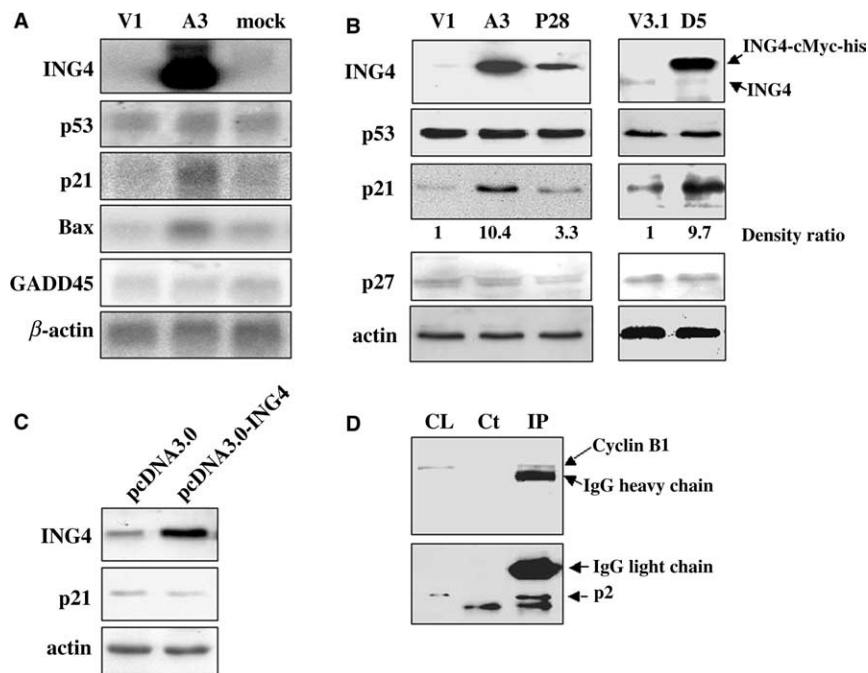


Fig. 4. The ING4-induced expression of some genes involved in cell cycle and apoptosis in the derived HepG2 cell clones. (A) Northern blot analysis showed the effect of ING4 on the transcriptional expressions of p53, p21, Bax and GADD45. Mock: the stable HepG2 cell clone with no expression of exogenous ING4 protein after pcDNA3.0-ING4 transfection and G418 selection. (B) The expression of p53, p21 and p27 proteins in the HepG2 cell clones. The protein lysates were analyzed by western blotting with anti-p53 antibody DO-1, anti-p21 antibody and anti-p27 antibody (Santa Cruz). (C) p53-deficient Saos-2 cells were transfected with pcDNA3.0-ING4 and control vector. The expression of p21 was detected by western blotting. (D) p21 associated with cyclin B1 in HepG2 cells. 400 μ g cell lysate from HepG2 cells was incubated with anti-p21 antibody and agarose-linked protein A/G (Santa Cruz), and then the immunoprecipitate was analyzed by western blotting with anti-cyclin B1 antibody and anti-p21 antibody, respectively. Precipitate from incubating the same cell lysates with agarose-linked protein A/G alone was used as negative control. CL, cell lysate; Ct, negative control; IP, Immunoprecipitation.

parallel with levels of ING4 protein and extent of G2/M arrest in the stable HepG2 subclones. In contrast to p21, p27^{KIP1}, which is a member of Cip/Kip family belonging to cyclin kinase inhibitors that are not regulated by p53, still maintained the same level in the HepG2 clones with exogenous ING4 as that in control cells (Fig. 4B). The data implied that ING4 could modulate the p53-inducible genes such as p21 and BAX, but not GADD45, in a p53-dependent manner. To strengthen the hypothesis, p53-deficient Saos-2 cells were employed to determine whether the p21 could be regulated by exogenous ING4 in p53-independent manner. The result showed that the overexpression of ING4 alone could not lead the upregulation of p21 in the p53-deficient Saos-2 cells (Fig. 4C), suggesting that p53-dependent pathway triggered by ING4 could play an important role in the upregulation of p21. However, except for p53 pathway, p21 could also be regulated through p53-independent pathways. Recently, ING4 was reported to interact with p300 and p65 (RelA) subunit of NF- κ B [17,18], suggesting that ING4 could be involved in the p53-independent signaling pathways, and ING4-induced upregulation of p21 and Bax still needs to be further studied.

To explore the molecular mechanisms of G2/M arrest, we analysed the levels of cyclin B1 and CDC2 in the stable HepG2 cell clones with exogenous ING4, since the cyclin B1/CDC2

complex is a known important regulator of G2/M transition. However, the overexpression of ING4 did not regulate the expression of cyclin B1 and CDC2 in HepG2 cells (supplementary Fig. S3). Therefore, the regulation of cyclin B1 and CDC2 expression could not be a major contributor to the ING4-induced G2/M arrest in HepG2 cells. Previous studies showed that p21 can inhibit CDC2 activity of cyclin B1/CDC2 complex and induced G2 arrest in various cells through binding to cyclin B1/CDC2 complex [22–24]. Herein, co-immunoprecipitation experiment revealed that cyclin B1 could physically associate with p21 in HepG2 cells (Fig. 4D), implicating that the ING4-induced upregulation of p21 could strengthen the association of cyclin B1 and p21, which may contribute to the G2/M arrest in the HepG2 cells.

3.5. ING4 could enhance the chemosensitivity to DNA-damage agents

Both ING1 and ING2 have been reported to enhance the chemosensitivity to DNA-damage agents in various human cells [12,13,25,26]. To evaluate the potential effects of ING4 on DNA damage, ETO and DOX were used to treat the HepG2 cells. ETO and DOX could induce the increasing expression of p53, but not result in the significant change of ING4 expression in HepG2 cells (Fig. 5A). However, the cell cycle analysis

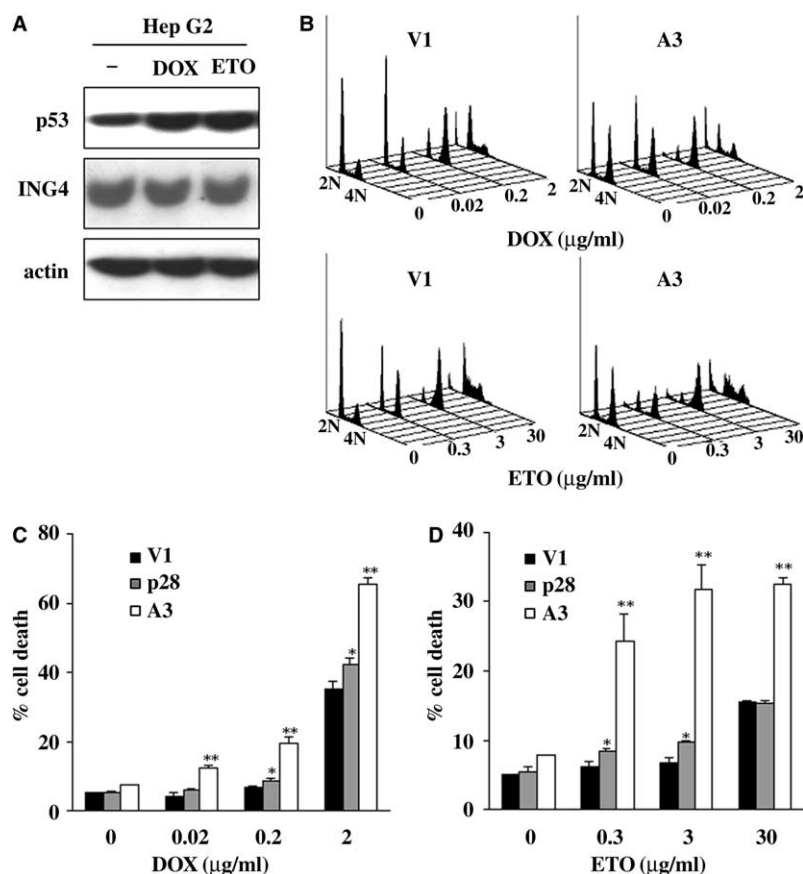


Fig. 5. ING4 could enhance the chemosensitivity to DNA-damage agents in HepG2 cells. (A) HepG2 cells were treated with 2 μg/ml DOX and 30 μg/ml ETO for 4 h. The expression of p53 and ING4 was detected by western blotting. (B) Flow cytometry analysis on the HepG2 clones, V1 and A3, was performed after treated with DOX and ETO for 48 h. The cell population of subdiploid peak before G1 phase was considered as death cells. (C) HepG2 cells (A3 and P28) with ING4 overexpression could be facilitated into death as exposure to DOX for 48 h. The data were representatives of three independent experiments from flow cytometry analysis. (D) Similar experiments indicated that the recombinant HepG2 cells could be more sensitive to ETO, particularly in A3 clone. (* $P < 0.05$, ** $P < 0.01$) The means and S.D. are included.

displayed the significant increase of the cell death in subdiploid peak in A3 cells with ING4 overexpression, as the HepG2 cells were treated with ETO or DOX, (Fig. 5B). As exposure to 0.02 µg/ml DOX, there were 13% death cells in A3 clone while only 4% in control clone V1. When DOX was increased to 0.2 µg/ml, about 20% A3 cells were dead but only 7% in V1 cells, indicating that the chemosensitivity to DOX in A3 cells was elevated up to about 3-folds (Fig. 5C). More interestingly, as exposure to 0.3 and 3 µg/ml of ETO, about 24% and 32% A3 cells could be dead while only 6% and 7% in V1 cells, respectively, implying that ING4 could enhance the chemosensitivity to ETO, up to 4–4.6-folds, which was more significant than that to DOX (Fig. 5D). Similarly, the somewhat increased cell death also was occurred in P28 clone with slight ING4 overexpression as exposure to the same DNA-damage agents, although weaker than that of A3 cells ($P < 0.05$, Fig. 5C and D). These results revealed that ING4 could enhance the cell death triggered by DNA-damage agents in HepG2 cells, although the expression of ING4 has no obvious response to DNA-damage.

Taken together, this study suggested that the ING4 gene, as a novel member of ING family, could negatively regulate cell proliferation by inducing G2/M arrest that might be correlated with upregulation of p21 induced by ING4; and, moreover, enhanced apoptosis induced by serum starvation in HepG2 cells. Interestingly, ING4 could also enhance the chemosensitivity to DNA-damage agents, implying that ING4 could be a candidate for gene therapy, in combination with chemotherapeutics.

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